

Appl. No. : 09/662,454  
Filed : September 14, 2000

### AMENDMENTS TO THE CLAIMS

Please cancel Claim 43 without prejudice, and amend Claims 42, 48, 51, 53, and 55-57 as follows:

1-41 (Canceled)

42. (Currently Amended) A ~~pharmaceutical~~ composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the amino acid sequence of SEQ ID NO: 3.

43. (Canceled)

44. (Canceled)

45. (Previously Presented) The composition of claim 42, wherein the nucleic acid molecule comprises the sequence of SEQ ID NO: 4.

46. (Withdrawn) The composition of claim 42, wherein the molecule encodes the amino acid sequence of SEQ ID NO: 1 shown in Figures 4G-4H.

47. (Withdrawn) The composition of claim 42, wherein the molecule comprises the nucleic acid sequence of SEQ ID NO: 2 shown in Figures 4A-4F.

48. (Currently Amended) A ~~pharmaceutical~~ composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the sequence of SEQ ID NO: 3, and wherein a portion of said nucleic acid molecule which encodes the structural region of hepatitis C virus has been replaced with a portion of a nucleic acid molecule of a different hepatitis C virus strain that encodes the corresponding structural region.

49. (Withdrawn) The composition according to claim 48, wherein the molecule encodes the amino acid sequence of SEQ ID NO: 5 shown in Figures 16G-16H.

50. (Withdrawn) The composition according to claim 48, wherein the molecule comprises the nucleic acid sequence of SEQ ID NO: 6 shown in Figures 16A-16F.

51. (Currently Amended) A ~~pharmaceutical~~ composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the sequence of SEQ ID NO: 3, and wherein a portion of the nucleic acid molecule which encodes at least one HCV protein has been replaced with a portion

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- of the genome of another hepatitis C virus strain which encodes the corresponding HCV protein.
52. **(Previously Presented)** The composition of claim 51, wherein the HCV protein is selected from the group consisting of: NS3 protease, E1 protein, E2 protein and NS4 protein.
53. **(Currently Amended)** A ~~pharmaceutical~~ composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the sequence of SEQ ID NO: 3, wherein a portion of the molecule encoding all or part of an HCV protein has been deleted, and wherein the HCV protein is selected from the group consisting of: P7, NS4B and NS5A proteins.
54. **(Canceled)**
55. **(Currently Amended)** A method for inducing an immune response ~~of immunizing an animal against hepatitis C virus~~ comprising the administration to an animal an effective amount of the composition ~~of the pharmaceutical composition~~ of claim 42, 48, 51 or 53 to induce an immune response ~~in an amount effective to induce immunity against hepatitis C virus~~.
56. **(Currently Amended)** The method according to claim 55, wherein the ~~pharmaceutical~~ composition is provided to an animal not infected with a hepatitis C virus prophylactically.
57. **(Currently Amended)** The method according to claim 55, wherein the ~~pharmaceutical~~ composition is provided to an animal infected with a hepatitis C virus.

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### **REMARKS**

Claims 42, 45-53, and 55-57 are pending. Claim 43 has been canceled without prejudice, and Claims 1-41, 44, and 54 were previously canceled. Claims 46, 47, 49, and 50 are withdrawn from consideration as being drawn to non-elected inventions. Claims 42, 45, 48, 51-53, and 55-57 are being examined. Applicant has amended Claims 42, 48, 51, 53, and 55-57 as being directed to a composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the amino acid sequence of SEQ ID NO: 3, and a method for inducing an immune response comprising the administration to an animal an effective amount of said composition to induce an immune response, and related compositions and methods. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

#### **A. Compliance with 35 USC 112, second paragraph**

As a preliminary matter, the Patent Office rejected Claims 42, 43, 45, 48, 51-53, and 55-57 under 35 USC 112, second paragraph, as being indefinite. Under MPEP 2173.02, the claims must be definite. Each of the claims was rejected as being indefinite in reciting the phrase "...said nucleic acid encoding a human hepatitis C virus having the amino acid sequence of SEQ ID NO:3..." It was unclear to the Patent Office how a viral particle that comprises elements obtained from a polypeptide comprising SEQ ID NO:3 can itself comprise SEQ ID NO:3. In response, the claims have been amended to make explicit what was implied, that said nucleic acid molecule encode a human hepatitis C virus polypeptide having the sequence of SEQ ID NO: 3. Under this amendment, the language of the claims explicitly states rather than implies definitive scope.

#### **B. Compliance with 35 USC 112, first paragraph**

The sole remaining issue is that the Patent Office rejected Claims 42, 43, 45, 48, 51-53, and 55-57 under 35 USC 112, first paragraph, as lacking enablement. According to MPEP 2164.08, enablement must be commensurate in scope with the claims. The rejection was

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originally applied to Claims 43, and 55-57, the method claims. Upon amendment of Claims 42, 45, 48, and 51-53 to add the feature "pharmaceutical" to the composition claims, the rejection was extended to the composition claims. By this amendment, the feature "pharmaceutical" has been deleted from the composition claims. Additionally, by this amendment, the claims are directed to a composition comprising a purified and isolated nucleic acid molecule, said nucleic acid molecule encoding a human hepatitis C virus polypeptide having the amino acid sequence of SEQ ID NO: 3, and a method for inducing an immune response comprising the administration to an animal an effective amount of said composition to induce an immune response, and related compositions and methods. The patent specification demonstrates construction of an infectious clone of strain H77, which is genotype 1a (Spec. at Figs 1-3) and strain HC-J4, which is genotype 1b (Spec. at Figs. 5-13). See also the post-filing date art that published these results as Yanagi et al., Proc. Natl. Acad. Sci. USA 94:8738, 1997 (Exhibit 1), and Yanagi et al., Virology 244:161, 1998 (Exhibit 2). Although the ORF of the latter clone was from strain HC-J4, most of the 5' and 3' terminal sequence originated from strain H77, thus the latter clone was a chimera of genotypes 1a and 1b (Spec. at Fig. 11). The infectivity of the HCV clones was determined by in vivo transfection: viral nucleic acid was injected directly into the liver of chimpanzees, the transfection protocol being by laparotomy of RNA transcripts of strain H77 (Spec. at 40:21-22), and by percutaneous intrahepatic injection of RNA transcripts of strain HC-J4 (Spec. at 53:2-5). The conclusion was that genetically stable infectious clones of HCV could be constructed from both important genotype strains of HCV (Spec. at Exs. 4 and 8). Anti-HCV antibodies were detected in chimpanzees following transfection with the infectious cDNA clone of strain H77 (Spec. at Fig. 18B and Ex. 4A) and the infectious cDNA clone of strain HC-J4 (Yanagi et al. 1998 at Fig. 9). Albeit inconvenient, chimpanzees represent the quintessential animal model for HCV infection. (Yanagi et al. 1997, p. 8738, col. 2, ¶ 2; and Yanagi et al. 1998, p. 162, col. 1, ¶ 2). These data provide evidence that genetic immunization of hepatitis C virus transcripts induces an immune response. The scope of the claims being co-extensive with a method for inducing an immune response, enablement is commensurate in scope with the claims.

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### CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 9/22/03

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AMEND  
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## Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee

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Contributed by Robert H. Purcell, June 9, 1997

**ABSTRACT** We have succeeded in constructing a stable full-length cDNA clone of strain H77 (genotype 1a) of hepatitis C virus (HCV). We devised a cassette vector with fixed 5' and 3' termini and constructed multiple full-length cDNA clones of H77 in a single step by cloning of the entire ORF, which was amplified by long reverse transcriptase-PCR, directly into this vector. The infectivity of two complete full-length cDNA clones was tested by the direct intrahepatic injection of a chimpanzee with RNA transcripts. However, we found no evidence for HCV replication. Sequence analysis of these and 16 additional full-length clones revealed that seven clones were defective for polyprotein synthesis, and the remaining nine clones had 6–28 amino acid mutations in the predicted polyprotein compared with the consensus sequence of H77. Next, we constructed a consensus chimera from four of the full-length cDNA clones with just two ligation steps. Injection of RNA transcripts from this consensus clone into the liver of a chimpanzee resulted in viral replication. The sequence of the virus recovered from the chimpanzee was identical to that of the injected RNA transcripts. This stable infectious molecular clone should be an important tool for developing a better understanding of the molecular biology and pathogenesis of HCV.

Hepatitis C virus (HCV) is the most important cause of transfusion-associated and community-acquired non-A, non-B hepatitis (1, 2). The infection is characterized by a high rate of chronicity (>80%) (1), and it is estimated that about 4 million people in the United States and more than 100 million people worldwide are chronically infected with HCV (2). These individuals have a high risk of developing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). Thus, HCV is a major cause of morbidity and mortality worldwide, and there is an urgent need for prevention of infection and for effective treatment. However, there currently is no vaccine for HCV, and therapy with interferon, which is the only antiviral drug with proven efficacy for treatment of chronic hepatitis C, is effective in only 20–30% of patients (3).

HCV has been tentatively classified in a separate genus (*Hepacivirus*) of the *Flaviviridae* family. Like members of the genera of *Flavivirus* and *Pestivirus*, HCV has a positive-sense single-stranded RNA genome that is about 9.5 kb in length (4). It consists of a highly conserved 5' untranslated region (UTR) of approximately 340 nt, a single long ORF of approximately 9,000 nt, and a unique 3' UTR of 200–300 nt. The 3' UTR contains three distinct regions consisting of a short variable sequence (variable region) adjoining a poly U-UC region of variable length followed by a highly conserved terminal sequence (conserved region) of approximately 100 nt (5–8). The ORF of HCV encodes a large polyprotein precursor that is cotranslationally and post-

translationally cleaved into several structural and nonstructural proteins (9).

The genome of HCV is highly heterogeneous, and many genetic groups have been described (10). The most divergent HCV isolates differ from each other by more than 30% over the entire genome. Moreover, HCV circulates as a quasispecies of closely related genomes in a single infected individual.

At present the chimpanzee represents the only animal model for HCV infection (11, 12). However, the availability of this animal model is very limited. Furthermore, HCV replication is not efficient in cultured cells *in vitro* (13). The difficulties in propagating HCV have hindered basic research and the development of antiviral therapies and vaccines.

The genome of positive-strand RNA viruses functions as mRNA from which all viral proteins necessary for virus propagation are translated. Thus, genomic RNA, as well as RNA transcripts from full-length cDNA clones, should be infectious. In the *Flaviviridae* family, infectious transcripts of full-length cDNAs have been described for flaviviruses (14–18) and pestiviruses (19–23). An infectious molecular clone of HCV would be an important tool for better understanding of its molecular biology and pathogenesis. Given the extensive genotype diversity of HCV, it also may be important to have infectious clones representative of more than one genotype or variant. Therefore, it is important to devise a strategy for efficiently constructing such clones. Recently, by using the long reverse transcriptase-PCR (RT-PCR) method we succeeded in the synthesis of 7.5-kb DNA amplicons of hepatitis A virus from which infectious RNA was transcribed (24). We furthermore demonstrated that it was possible to amplify the near full-length HCV genome in a single round of long RT-PCR (25). In the present study, we used this methodology to perform a detailed sequence analysis of the H77 strain of HCV. The sequence data were used to construct a cassette vector into which products from long RT-PCR were efficiently inserted to provide full-length genomic cDNA clones that were tested for their ability to yield infectious RNA transcripts.

### MATERIALS AND METHODS

**Virus Stock.** Plasma containing strain H77 of HCV was obtained from a patient in the acute phase of transfusion-associated non-A, non-B hepatitis (11). Strain H77 belongs to genotype 1a of HCV (26, 27). The consensus sequence for most of its genome has been determined (7, 26–28).

Abbreviations: HCV, hepatitis C virus; UTR, untranslated region; RT-PCR, reverse transcriptase-PCR; GE, genome equivalents; p.i., postinoculation.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF011751–AF011753).

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**RNA Purification.** Total RNA from 10  $\mu$ l of H77 plasma was extracted with the TRIzol system (GIBCO/BRL). The RNA pellet was resuspended in 100  $\mu$ l of 10 mM DTT with 5% (vol/vol) RNasin (20–40 units/ $\mu$ l) (Promega), and 10- $\mu$ l aliquots were stored at  $-80^{\circ}\text{C}$ . In subsequent experiments RT-PCR was performed on RNA equivalent to 1  $\mu$ l of H77 plasma, which contained an estimated  $10^5$  genome equivalents (GE) of HCV (29).

**Primers.** Primers were deduced from the genomic sequences of strain H77 previously published (see above) or determined in this study (Table 1). Primers for long RT-PCR were size-purified.

**cDNA Synthesis.** The RNA was denatured at  $65^{\circ}\text{C}$  for 2 min, and cDNA synthesis was performed in a 20- $\mu$ l reaction volume with Superscript II reverse transcriptase (GIBCO/BRL) at  $42^{\circ}\text{C}$  for 1 hr using specific antisense primers as described previously (24). The cDNA mixture was treated with RNase H and RNase T1 (GIBCO/BRL) for 20 min at  $37^{\circ}\text{C}$ .

**Amplification and Cloning of the 3' UTR.** The 3' UTR of strain H77 was amplified by PCR in two different assays. In both of these nested PCR reactions the first round of PCR was performed in a total volume of 50  $\mu$ l in 1 $\times$  buffer, 250  $\mu$ M each deoxynucleoside triphosphate (Pharmacia), 20 pmol each external sense and antisense primers, 1  $\mu$ l of the Advantage KlenTaq polymerase mix (CLONTECH), and 2  $\mu$ l of the final cDNA reaction mixture. In the second round of PCR, 5  $\mu$ l of the first-round PCR mixture was added to 45  $\mu$ l of PCR mixture prepared as described above. Each round of PCR (35 cycles), which was performed in a DNA thermal cycler 480 (Perkin-Elmer), consisted of denaturation at  $94^{\circ}\text{C}$  for 1 min (in first cycle 1 min 30 sec), annealing at  $60^{\circ}\text{C}$  for 1 min, and elongation at  $68^{\circ}\text{C}$  for 2 min. In one experiment a region from NS5B to the conserved region of the 3' UTR was amplified with the external primers H9261F and H3'X58R, and the internal primers H9282F and H3'X45R (Table 1). In another experiment, a region from the variable region to the very end of the 3' UTR was amplified with the external primers H9375F and H3'X-35R, and the internal primers H9386F and H3'X-38R (Table 1, Fig. 1). Amplified products were purified with QIAquick PCR purification kit (Qiagen), digested with *Hind*III and *Xba*I (Promega), purified by either gel electrophoresis or phenol/chloroform extraction, and then cloned into the multiple cloning site of pGEM-9zf(–) (Promega) or pUC19 (Pharmacia). Cloning of cDNA into the vector was performed with T4 DNA ligase (Promega) by standard procedures.

**Amplification of Near Full-Length H77 Genomes by Long PCR.** The reactions were performed in a total volume of 50  $\mu$ l in 1 $\times$  buffer, 250  $\mu$ M each deoxynucleoside triphosphate, 10 pmol of sense and antisense primers each, 1  $\mu$ l of the Advantage KlenTaq polymerase mix, and 2  $\mu$ l of the cDNA reaction mixture (24, 25). A single PCR round of 35 cycles was performed in a Robocycler thermal cycler (Stratagene) and consisted of denaturation at  $99^{\circ}\text{C}$  for 35 sec, annealing at  $67^{\circ}\text{C}$  for 30 sec, and

elongation at  $68^{\circ}\text{C}$  for 10 min during the first five cycles, 11 min during the next 10 cycles, 12 min during the following 10 cycles, and 13 min during the last 10 cycles. To amplify the complete ORF of HCV by long RT-PCR we used the sense primers H1 or A1 deduced from the 5' UTR and the antisense primer H9417R deduced from the variable region of the 3' UTR (Table 1, Fig. 1).

**Construction of Full-Length H77 cDNA Clones.** The long PCR products amplified with H1 and H9417R primers were cloned directly into pGEM-9zf(–) after digestion with *Not*I and *Xba*I (Promega) (Fig. 1). We obtained only two clones with inserts of the expected size, pH21<sub>1</sub> and pH50<sub>1</sub>. Next, the chosen 3' UTR was cloned into both pH21<sub>1</sub> and pH50<sub>1</sub> after digestion with *Afl*III and *Xba*I (New England Biolabs). DH5 $\alpha$  competent cells (GIBCO/BRL) were transformed and selected with Luria-Bertani agar plates containing 100  $\mu$ g/ml ampicillin (Sigma). Then the selected colonies were cultured in Luria-Bertani liquid containing ampicillin at  $30^{\circ}\text{C}$  for 18–20 hr (transformants containing full-length or near full-length cDNA of H77 produced a very low yield of plasmid when cultured at  $37^{\circ}\text{C}$  or for more than 24 hr). After small-scale preparation (Wizard Plus Minipreps DNA Purification Systems, Promega) each plasmid was retransformed to select a single clone, and large-scale preparation of plasmid DNA was performed with a Qiagen plasmid Maxi kit.

**Cloning of Long RT-PCR Products into a Cassette Vector.** To improve the efficiency of cloning, we constructed a vector with consensus 5' and 3' termini of HCV strain H77 (Fig. 1). This cassette vector (pCV) was obtained by cutting out the *Bam*HI fragment (nucleotides 1,358–7,530 of the H77 genome) from pH50, followed by religation. Next, the long PCR products of H77 amplified with H1 and H9417R or A1 and H9417R primers were purified (GeneClean spin kit; BIO 101) and cloned into pCV after digestion with *Age*I and *Afl*III (New England Biolabs) or with *P*inAI (isoschizomer of *Age*I) and *B*frI (isoschizomer of *Afl*III) (Boehringer Mannheim). Large-scale preparations of the plasmids containing full-length cDNA of H77 were performed as described above.

**Construction of H77 Consensus Chimeric cDNA Clone.** To construct a full-length cDNA clone of H77 with an ORF encoding the consensus amino acid sequence, we made a chimera from four of the cDNA clones obtained above. This consensus chimera, pCV-H77C, was constructed in two ligation steps by using standard molecular procedures and convenient cleavage sites and involved first a two-piece ligation and then a three-piece ligation. Large-scale preparation of pCV-H77C was performed as described above.

**In Vitro Transcription.** Plasmids containing the full-length HCV cDNA were linearized with *Xba*I (Promega) and purified by phenol/chloroform extraction and ethanol precipitation. A 100- $\mu$ l reaction mixture containing 10  $\mu$ g of linearized plasmid DNA, 1 $\times$  transcription buffer, 1 mM ATP, CTP, GTP, and UTP, 10 mM DTT, 4% (vol/vol) RNasin (20–40 units/ $\mu$ l), and 2  $\mu$ l of T7 RNA polymerase (Promega) was incubated at  $37^{\circ}\text{C}$  for 2 hr.

Table 1. Oligonucleotides used for PCR amplification of strain H77 of HCV

Designation	Sequence (5' $\rightarrow$ 3')*
H9261F	GGCTACAGCGGGGAGACATTTATCACAGC
H3'X58R	TCATGCGGCTCACGGACCTTTCACAGCTAG
H9282F	<b>GTCCAAGCTT</b> TATCACAGCGTGTCTCATGCCCGGCCCCG
H3'X45R	<b>CGTCTCTAG</b> AGGACCTTTCACAGCTAGCCGTGACTAGGG
H9375F	TGAAGGTTGGGGTAAACACTCCGGCCTCTTAGGCCATT
H3'X-35R	ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTC
H9386F	<b>GTCCAAGCTT</b> ACGGCTAAACACTCCGGCCTCTTAAGCCATTTCCTG
H3'X-38R	<b>CGTCTCTAG</b> ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGC
H1	<b>TTTTTTT</b> TCGGCGCGCTAATACGACTCACTATAGCCGCCCCCTGATGGGGGCGACACTCCACCATG
A1	ACTGTCTTACGCGAGAAAGCGTCTAGCCAT
H9417R	<b>CGTCTCTAG</b> CAGGAAATGGCTTAAGAGGCCGGAGTGTTTACC

\*HCV sequences are shown in lightface text, non-HCV-specific sequences are shown in boldface, and artificial cleavage sites used for cDNA cloning are underlined. The core sequence of the T7 promoter in primer H1 is shown in italics.

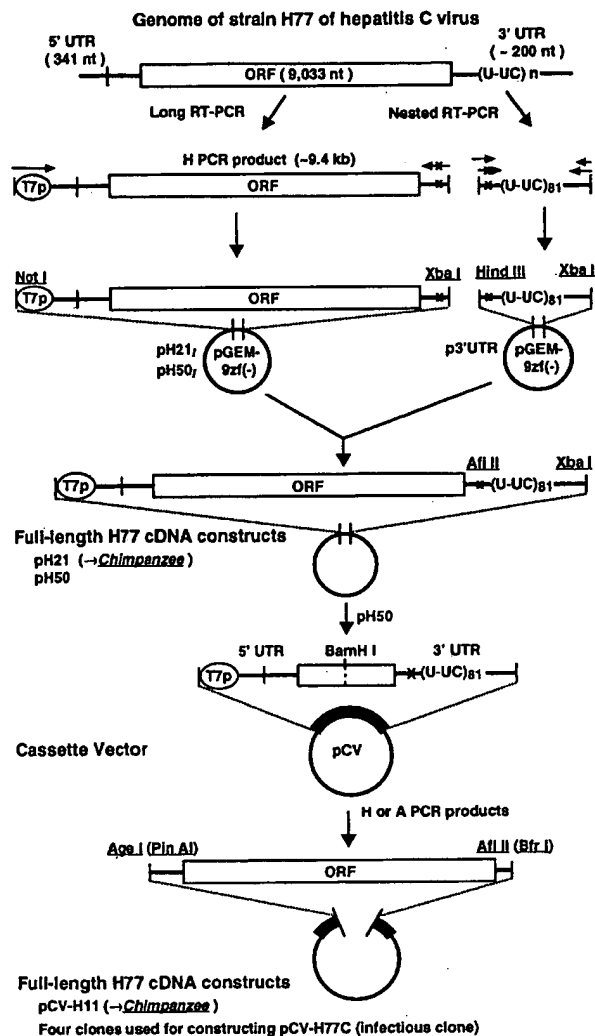


FIG. 1. Strategy for the construction of full-length cDNA clones of HCV strain H77. The long PCR products amplified with H1 and H9417R primers were cloned directly into pGEM-9zf(-) after digestion with *NotI* and *XbaI* (pH21 and pH50). Next, the 3' UTR was cloned into both pH21 and pH50 after digestion with *AflII* and *XbaI* (pH21 and pH50). pH21 was tested for infectivity in a chimpanzee. To improve the efficiency of cloning, we constructed a cassette vector with consensus 5' and 3' termini of H77. This cassette vector (pCV) was obtained by cutting out the *BamHI* fragment (nucleotides 1,358–7,530 of the H77 genome) from pH50, followed by religation. Finally, the long PCR products of H77 amplified with primers H1 and H9417R (H product) or primers A1 and H9417R (A product) were cloned into pCV after digestion with *AgeI* and *AflII* or with *PinAI* and *BfrI*. The latter procedure yielded multiple complete cDNA clones of strain H77 of HCV.

Five microliters of the reaction mixture was analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The transcription reaction mixture was diluted with 400  $\mu$ l of ice-cold PBS without calcium or magnesium, immediately frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . The final nucleic acid mixture was injected into chimpanzees within 24 hr.

**Intrahepatic Transfection of Chimpanzees.** Laparotomy was performed, and aliquots from two transcription reactions were injected into six sites of the exposed liver (30). Serum samples were collected weekly from chimpanzees and monitored for liver enzyme levels and anti-HCV antibodies. Weekly samples of 100  $\mu$ l of serum were tested for HCV RNA in a highly sensitive nested RT-PCR assay with AmpliTaq Gold (Perkin-Elmer) (29, 31). The genome titer of HCV was

estimated by testing 10-fold serial dilutions of the extracted RNA in the RT-PCR assay (29). The two chimpanzees used in this study were maintained under conditions that met all requirements for their use in an approved facility.

The consensus sequence of the complete ORF from HCV genomes recovered at week 2 postinoculation (p.i.) was determined by direct sequencing of PCR products obtained in long RT-PCR with primers A1 and H9417R followed by nested PCR of 10 overlapping fragments. The consensus sequence of the variable region of the 3' UTR was determined by direct sequencing of an amplicon obtained in nested RT-PCR as described above. Finally, we amplified selected regions independently by nested RT-PCR with AmpliTaq Gold.

**Sequence Analysis.** Both strands of DNA from PCR products, as well as plasmids, were sequenced with the Applied Biosystems PRISM Dye Termination Cycle Sequencing Ready Reaction Kit using AmpliTaq DNA polymerase (Perkin-Elmer) and about 100 specific sense and antisense sequence primers.

We determined the consensus sequence of HCV strain H77 in two different ways. In one approach we directly sequenced overlapping PCR products amplified in nested RT-PCR from the H77 plasma sample. The sequence analyzed (nucleotides 35–9,417) included the entire genome except the very 5' and 3' termini. In the second approach, the consensus sequence of nucleotides 157–9,384 was deduced from the sequences of 18 full-length cDNA clones.

## RESULTS

**Variability in the Sequence of the 3' UTR of HCV Strain H77.** The heterogeneity of the 3' UTR was analyzed by cloning and sequencing of DNA amplicons obtained in nested RT-PCR. We analyzed 19 clones, which included sequences of the entire variable region, the poly U-UC region, and the adjacent 19 nt of the conserved region, and 65 clones containing sequences of the entire poly U-UC region and the first 63 nts of the conserved region. We confirmed that the variable region consisted of 43 nts, including two conserved termination codons (32). The sequence of the variable region was highly conserved within H77 because only three point mutations were found among the 19 clones analyzed. A poly U-UC region was present in all 84 clones analyzed. However, its length varied from 71–141 nts. The length of the poly U region was 9–103 nts, and that of the poly UC region was 35–85 nts. The number of C residues increased toward the 3' end of the poly UC region but the sequence of this region was not conserved. The first 63 nts of the conserved region were highly conserved among the clones analyzed, with a total of only 14 point mutations. To confirm the validity of the analysis, we reamplified the 3' UTR directly from a full-length cDNA clone of HCV (see below) by the nested-PCR procedure with the primers in the variable region and at the very 3' end of the HCV genome and cloned the PCR product. Eight clones had 1–7 nt deletions in the poly U region. Furthermore, although the C residues of the poly UC region were maintained, the spacing of these varied because of 1–2 nt deletions of U residues. These deletions must be artifacts introduced by PCR, and such mistakes may have contributed to the heterogeneity originally observed in this region. However, the conserved region of the 3' UTR was amplified correctly, suggesting that the deletions were due to difficulties in transcribing a highly repetitive sequence.

One of the 3' UTR clones was selected for engineering of full-length cDNA clones of H77. This clone had the consensus variable sequence except for a single point mutation introduced to create an *AflII* cleavage site, a poly U-UC stretch of 81 nts with the most commonly observed UC pattern and the consensus sequence of the complete conserved region of 101 nt, including the distal 38 nt that originated from the antisense primer used in the amplification. After linearization with *XbaI*, the DNA template of this clone had the authentic 3' end.



**The Entire ORF of H77 Amplified in One Round of Long RT-PCR.** We previously demonstrated that a 9.25-kb fragment of the HCV genome from the 5' UTR to the 3' end of NS5B could be amplified from 10<sup>4</sup> GE of H77 by a single round of long RT-PCR (25). In the current study, by optimizing primers and cycling conditions, we were able to amplify the entire ORF of H77 in a single round of long RT-PCR with primers from the 5' UTR and the variable region of the 3' UTR. In fact, we could amplify 9.4 kb of the H77 genome (H product: from the very 5' end to the variable region of the 3' UTR) from 10<sup>5</sup> GE or 9.3 kb (A product: from within the 5' UTR to the variable region of the 3' UTR) from 10<sup>4</sup> GE or 10<sup>5</sup> GE, in a single round of long RT-PCR (Fig. 2). The PCR products amplified from 10<sup>5</sup> GE of H77 were used for engineering full-length cDNA clones (see below).

**Construction of Multiple Full-Length cDNA Clones of H77 in a Single Step by Cloning of Long RT-PCR Amplicons Directly into a Cassette Vector with Fixed 5' and 3' Termini.** We first attempted direct cloning of the long PCR products (H), which contained a 5' T7 promoter, the authentic 5' end, the entire ORF of H77 and a short region of the 3' UTR, into pGEM-9zf(-) vector by *NotI* and *XbaI* digestion. However, among the 70 clones examined all but two had inserts that were shorter than predicted. Sequence analysis identified a second *NotI* site in the majority of clones, which resulted in deletion of the nucleotides past position 9,221. Only two clones (pH21<sub>i</sub> and pH50<sub>i</sub>) were missing the second *NotI* site and had the expected 5' and 3' sequences of the PCR product. Therefore, full-length cDNA clones (pH21 and pH50) were constructed by inserting the chosen 3' UTR into pH21<sub>i</sub> and pH50<sub>i</sub>, respectively. Sequence analysis revealed that clone pH21 had a complete full-length sequence of H77; this clone was tested for infectivity. The second clone, pH50, had 1 nt deletion in the ORF at position 6,365; this clone was used to make a cassette vector.

Because we could not amplify the entire genome in one step but we could amplify the complete ORF, we constructed a cassette vector with fixed 5' and 3' termini as an intermediate of full-length cDNA clones. We constructed this vector (pCV) by digestion of clone pH50 with *Bam*HI, followed by religation. This shortened the plasmid so that it could be readily distinguished from plasmids containing the full-length insert. Attempts to clone long RT-PCR products (H) into pCV by *AgeI* and *Afl*III yielded only 1 of 23 clones with an insert of the expected size. To increase the efficiency of cloning, we re-

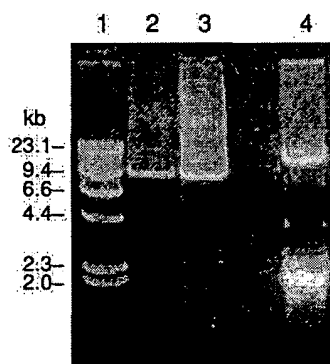


FIG. 2. Gel electrophoresis of long RT-PCR amplicons of the entire ORF of H77 and the transcription mixture of the infectious clone of H77. The complete ORF was amplified by long RT-PCR with the primers H1 or A1 and H9417R from 10<sup>5</sup> GE of H77. A total of 10  $\mu$ g of the consensus chimeric clone (pCV-H77C) linearized with *XbaI* was transcribed in a 100- $\mu$ l reaction with T7 RNA polymerase. Five microliters of the transcription mixture was analyzed by gel electrophoresis and the remainder of the mixture was injected into a chimpanzee. Lane 1, molecular weight marker; lane 2, products amplified with primers H1 and H9417R; lane 3, products amplified with primers A1 and H9417R; lane 4, transcription mixture containing the RNA transcripts and linearized clone pCV-H77C (12.5 kb).

peated the procedure but used *Pin*AI and *Bf*I instead of the respective isoschizomers *Age*I and *Afl*III. By this protocol, 24 of 31 H clones and 30 of 35 A clones had the full-length cDNA of H77 as evaluated by restriction enzyme digestion. A total of 16 clones, selected at random, were each retransformed, and individual plasmids were purified and completely sequenced.

**Two Full-Length cDNA Clones of H77 Were Not Infectious in a Chimpanzee.** The infectivity of the two first complete full-length cDNA clones of H77 (pH21 and pCV-H11) we obtained were tested by the intrahepatic injection of a chimpanzee with RNA transcripts. The transcription mixture from each clone was injected into three sites of the exposed liver. However, we found no evidence for HCV replication in the chimpanzee. Weekly serum samples were negative for HCV RNA at weeks 1–17 p.i. in a highly sensitive nested RT-PCR assay. The cDNA template injected along with the RNA transcripts also was not detected in this assay. The chimpanzee remained negative for antibodies to HCV throughout follow-up. Thus, clones pH21 and pCV-H11 were not infectious.

**Failure to Identify a Consensus Clone by Sequence Analysis of the ORF of 18 Full-Length cDNA Clones.** We performed sequence analysis of 18 full-length clones. Clone pH21, which was not infectious (see above), had seven aa substitutions in the entire predicted polyprotein compared with the consensus sequence of H77 (Fig. 3). The most notable mutation, at position 1,026, changed L to Q, which altered the cleavage site between NS2 and NS3 (33). Clone pCV-H11, which also was not infectious (see above), had 21 aa substitutions in the predicted polyprotein compared with the consensus sequence of H77 (Fig. 3). One aa mutation (position 564) eliminated a highly conserved C residue in the E2 protein (34). These two mutations might conceivably account for the lack of infectivity of the two clones but any of the other mutations might have been responsible. Alternatively, the 5' and 3' termini might not have been viable.

The infectivity of the remaining 16 full-length clones was not tested. However, four clones had a single nucleotide deletion in the ORF of H77, which would result in a frame shift, and an additional clone had two nucleotide insertions in the ORF, which also would cause a frame shift. Finally, stop codons were identified in the ORF of two more clones. Thus, these seven clones were defective for polyprotein synthesis and clearly would not be infectious. This left us with nine potentially infectious clones. However, these clones had 6–28 aa mutations in the predicted polyprotein compared with the consensus sequence of H77. More importantly, each clone had aa mutations not observed in other isolates of HCV (10).

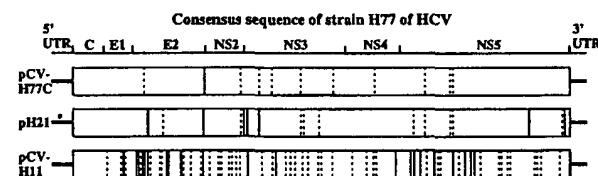


FIG. 3. Diagram of the genome organization of HCV strain H77 and the genetic heterogeneity of individual full-length clones compared with the consensus sequence of H77. Solid lines represent amino acid changes. Dashed lines represent silent mutations. A \* in pH21 represents a point mutation at nucleotide 58 in the 5' UTR. In the ORF, the consensus chimeric clone pCV-H77C had 11 nucleotide differences [at positions 1,625 (C  $\rightarrow$  T), 2,709 (T  $\rightarrow$  C), 3,380 (A  $\rightarrow$  G), 3,710 (C  $\rightarrow$  T), 3,914 (G  $\rightarrow$  A), 4,463 (T  $\rightarrow$  C), 5,058 (C  $\rightarrow$  T), 5,834 (C  $\rightarrow$  T), 6,734 (T  $\rightarrow$  C), 7,154 (C  $\rightarrow$  T), and 7,202 (T  $\rightarrow$  C)] and one amino acid change (F  $\rightarrow$  L at amino acid 790) compared with the consensus sequence of H77. This clone was infectious. Clone pH21 and pCV-H11 had 19 nucleotide (7 amino acid) and 64 nucleotide (21 amino acid) differences, respectively, compared with the consensus sequence of H77. These two clones were not infectious. A single point mutation in the 3' UTR at nucleotide 9,406 (G  $\rightarrow$  A) introduced to create an *Afl*III cleavage site is not shown.

**Transcripts from a cDNA clone Representing the Consensus Sequence of HCV Strain H77 Are Infectious When Transfected into the Liver of a Chimpanzee.** It is well established that RNA transcripts of cDNA clones encompassing the consensus sequences of flaviviruses or pestiviruses are infectious (14, 19). Given the limited availability of chimpanzees, which currently represent the only reliable model for HCV propagation, we decided to test the infectivity of a consensus clone of H77. A consensus chimera was constructed from four of the full-length cDNA clones with just two ligation steps. The final construct, pCV-H77C, had 11 nucleotide differences from the consensus sequence of H77 in the ORF (Fig. 3). However, 10 of these nucleotide differences represented silent mutations. The chimeric clone differed from the consensus sequence at only one amino acid (L instead of F at position 790). Among the 18 ORFs analyzed above, the F residue was found in 11 clones and the L residue in seven clones. However, the L residue was dominant in other isolates of genotype 1a, including a first passage of H77 in a chimpanzee (27). Thus, we did not believe that this amino acid difference was critical for infectivity.

To test the infectivity of the consensus chimeric clone of H77 we performed intrahepatic transfection of a chimpanzee. The pCV-H77C clone was linearized with *Xba*I and transcribed *in vitro* by T7 RNA polymerase (Fig. 2). The transcription mixture was next injected into six sites of the chimpanzee liver. We used exactly the same total amount of DNA template and transcription mixture as used in the unsuccessful transfection described above in which viral sequences were not detected at any time after injection. The chimpanzee became infected with HCV as measured by detection of  $10^2$  GE/ml of viral genome at week 1 p.i. Furthermore, the HCV titer increased to  $10^4$  GE/ml at week 2 p.i. and had reached  $10^6$  GE/ml by week 8 p.i.

We analyzed the sequence of the HCV genomes from the serum sample collected at week 2 p.i. The consensus sequence of nucleotides 298–9,375 of the recovered genomes was determined by direct sequencing of PCR products obtained in long RT-PCR followed by nested PCR of 10 overlapping fragments. The identity to clone pCV-H77C sequence was 100%. The consensus sequence of nucleotides 96–291, 1,328–1,848, 3,585–4,106, 4,763–5,113, and 9,322–9,445 was determined from PCR products obtained in different nested RT-PCR assays. The identity of these sequences with pCV-H77C was also 100%. These latter regions contained four mutations unique to the consensus chimera, including the artificial *A7II* cleavage site in the 3' UTR. Therefore, RNA transcripts of this clone of HCV were infectious.

## DISCUSSION

In the present study, we have constructed an infectious clone of strain H77 of HCV. This clone represents the consensus sequence of H77 and contains a 5' UTR of 341 nt, an ORF of 9,033 nt, and a 3' UTR of 225 nt (a variable region of 43 nt with a single point mutation, poly U-UC region of 81 nt, and a conserved region of 101 nt). Two other full-length cDNA clones of H77 with the same termini were not infectious, most likely due to one or more of the amino acid mutations found in the predicted polyprotein.

Due to the lack of reliable *in vitro* propagation systems of HCV we could not perform screening for infectivity in cell cultures. We previously established an *in vivo* transfection system for RNA transcripts of infectious clones of hepatitis A virus in tamarins (30), as well as in chimpanzees. Therefore, the infectivity of RNA transcripts of full-length HCV clones was tested by injecting transcription mixtures into the liver of chimpanzees.

We demonstrated that RNA transcripts of the consensus chimera of H77 were infectious *in vivo* and that the titer of genomic sequences recovered from the serum of the chimpanzee increased over time. Sequence analysis demonstrated that the recombinant virus recovered from the chimpanzee after the increase in viral titer had a sequence identical to that of the chimeric clone, including several unique mutations. It is possible that the cDNA injected with the transcribed RNA was itself

infectious. However, we previously demonstrated that the cDNA clone of hepatitis A virus, inserted into a similar vector also lacking eukaryotic promoters, was not infectious when injected into the liver of tamarins (30).

The viremic pattern observed in the early phase of the infection with the recombinant virus was similar to that observed in chimpanzees inoculated intravenously with strain H77 or other strains of HCV (ref. 12; J.B., unpublished data). Viral hepatitis normally develops after week 8 p.i. However, biological variation exists, and some chimpanzees develop only minimal evidence of hepatitis. Thus, a careful study of the phenotype of the infectious clone of HCV would require transfection of several chimpanzees.

Reports of infectious transcripts of full-length cDNAs have been published for several members of the genus *Flavivirus* (14–18) and more recently for members of the genus *Pestivirus* (19–23). Most of these infectious cDNA clones were constructed by screening of cDNA libraries followed by the assembly of clones representing the consensus sequence. These common procedures are complicated and time-consuming, particularly in the case of HCV, which exists as heterogeneous quasispecies. We previously showed that full-length infectious cDNA clones of hepatitis A virus could be engineered in one step by cloning of long RT-PCR amplicons (35). We had to modify this approach for HCV, because we were not able to amplify the entire genome by long RT-PCR, probably because of the strong predicted secondary structure, as well as the presence of a long poly U-UC region, in the 3' UTR. Instead we used a cassette vector with fixed termini of the HCV genome and cloned the entire ORF, amplified in long RT-PCR, directly into this vector. Thus, with this strategy we were able to construct full-length HCV clones in two steps.

One conclusion of our study is that a high proportion of HCV genomes probably are defective. We found that two of the complete full-length clones constructed were not infectious. Sequence analysis revealed that 7 of 16 additional clones were defective for polyprotein synthesis, and all clones had multiple amino acid mutations compared with the consensus sequence of the parent strain. We cannot rule out that some of these mutations were introduced in the RT-PCR and cloning procedures. However, the infectivity titer of H77 is 1–2  $\log_{10}$  lower than the genome titer suggesting that less than 10% of the genomes are infectious (refs. 11 and 29; R.H.P., unpublished data). Moormann *et al.* (19) reported that five aa changes (compared with the consensus sequence) in the polyprotein accounted for noninfectivity of classical swine fever virus. Furthermore, at a recent meeting† it was reported by C. M. Rice and A. A. Kolykhalov that, of numerous clones of H77 they tested, only those engineered to contain the consensus sequence were infectious. Although nine of our clones potentially could be infectious they all had six or more mutations compared with the consensus sequence of strain H77. Because of the limited availability of chimpanzees, we constructed a consensus chimeric clone of strain H77. By using four of the full-length clones we constructed this clone in just two steps.

The infectious nature of the consensus chimera indicated that the regions of the 5' and 3' UTRs incorporated into the cassette vector did not destroy viability and suggested that it indeed was one or more of the coding mutations in each of the original two constructs that were lethal. Therefore, it should be possible to use the cassette vector to construct infectious cDNA clones of other HCV strains but it probably will be necessary to insert the consensus sequence for each ORF tested.

The infectious clone of HCV generated in the present study contained the 5' and 3' termini previously published by others (7, 36). Because the core sequence of the T7 promoter was posi-

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tioned immediately before the authentic G of the 5' UTR sequence, the RNA transcripts should include the exact 5' end of HCV. Furthermore, after plasmid linearization with *Xba*I, the template DNA and therefore the RNA transcripts should end exactly at the 3' end. The 5' UTR of HCV previously has been found to contain an internal ribosomal entry site (4). We found that uncapped RNA was infectious, indicating that cap-independent translation initiation is used by HCV for gene expression. Thus, the internal ribosomal entry site must be functional *in vivo*. It is unclear which elements of the 3' UTR are required for infectivity of HCV. We found that the variable region was highly conserved in H77. However, the point mutation introduced in this region for cloning purposes was tolerated in the infectious clone. The length and sequence of the poly U-UC region varied greatly in H77. Our infectious clone of H77 contained a poly U-UC of intermediate length. Finally, we found that the conserved region of the 3' UTR was highly conserved, and our infectious clone contained the consensus sequence (101 nts) of this region (7). In one previous study it was reported that the RNA transcripts from a cDNA clone of HCV-1, the prototype of genotype 1a, resulted in a low level of replication after transfection into a human hepatoma cell line (37). This cDNA clone did not contain the conserved region of the 3' UTR. Furthermore, the infectivity of this clone was not tested *in vivo*. Recently, it was reported that deletions in the variable region of the 3' UTR of Dengue virus and Kunjin virus did not render these viruses noninfectious (38, 39). However, the conserved core sequences of the 3' UTR were critical for viral replication. Further studies, which are now possible, are needed to determine which regions of the 3' UTR are essential for HCV replication.

Instability of plasmids containing the full-length cDNA constructs of flaviviruses or pestiviruses created major obstacles to the construction of infectious clones for these viruses (14–23). We used the high copy vector pGEM-9zf(–) and DH5 $\alpha$  cells to prepare full- or near full-length cDNA clones of HCV. We found that transformants produced a very low yield of plasmid DNA when cultured at 37°C or for more than 24 hr. However, we could obtain a good yield of plasmids when cultures were incubated at 30°C for 18–20 hr. Furthermore, the sequence of the chimeric clone of H77, after retransformation and large-scale preparation, was identical to the sequence of the inserts from the four clones used in the construction. These results indicate that our infectious clone was stable in DH5 $\alpha$  cells.

The quasispecies nature of HCV is believed to be important for viral persistence (10). The successful infection of a chimpanzee with a single clone of HCV provides us with a unique opportunity to study the quasispecies nature of this virus and its evolution *in vivo*, as well as its importance for viral persistence.

In conclusion, we have constructed a genetically stable infectious clone of HCV. The approach used to engineer this clone should be applicable to construction of full-length infectious cDNAs of other HCV strains (10), as well as of a number of recently discovered related viruses (see ref. 40). Furthermore, this infectious clone might be helpful in developing effective *in vitro* propagation systems. Finally, the availability of an infectious clone of HCV makes it possible to study in detail the mechanisms of viral replication and pathogenesis.

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## Transcripts of a Chimeric cDNA Clone of Hepatitis C Virus Genotype 1b Are Infectious *in Vivo*

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We constructed a chimeric cDNA clone of hepatitis C virus (HCV) that is infectious. The chimeric genome encodes the polyprotein of a genotype 1b strain (HC-J4) of HCV and replicates via 5' and 3' untranslated regions of a genotype 1a strain. The infectivity of three full-length cDNA clones was tested by direct injection of RNA transcripts into the liver of a chimpanzee. The chimpanzee became infected with HCV and the viral titer increased over time from 10<sup>2</sup> genome equivalents (GE)/ml at week 1 postinoculation (p.i.) to 10<sup>4</sup>–10<sup>5</sup> GE/ml during weeks 3–11 p.i. Antibodies to HCV were detected from week 18 p.i. However, the chimpanzee did not develop hepatitis. Sequence analysis of PCR products amplified from the serum of the chimpanzee demonstrated that only one of the three clones was infectious. Sequence comparisons with the cloning source, an acute-phase infectious plasma pool derived from an experimentally infected chimpanzee, showed that this infectious clone had three amino acids that differed from the consensus sequence of HC-J4, whereas the two noninfectious clones had seven and nine amino acid differences, respectively. Together, genotype 1b, represented by the infectious molecular clone described herein, and genotype 1a, represented by the two cDNA clones previously shown to be infectious for chimpanzees, account for the majority of HCV infections in the United States, Europe, and Japan. © 1998 Academic Press

### INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease (Houghton, 1996). More than 80% of individuals infected with HCV become chronically infected, with about 4 million people infected in the United States (Alter, 1997; Hoofnagle, 1997). Chronically infected individuals have a relatively high risk of developing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Hoofnagle, 1997). The only effective therapy for chronic hepatitis C, interferon (IFN), induces a sustained response in less than 25% of treated patients (Fried and Hoofnagle, 1995). Consequently, HCV is currently the most common cause of end-stage liver failure and the reason for about 30% of liver transplants performed in the United States (Hoofnagle, 1997). There is no vaccine for HCV. Although the number of acute HCV infections has declined, in part because of effective screening of blood and blood products, it is estimated that there are still more than 25,000 new infections yearly in the United States (Alter, 1997). Thus, HCV constitutes a serious public health problem.

Hepatitis C virus has a positive-sense single-strand RNA genome and is a member of the virus family Flavi-

viridae (Choo *et al.*, 1991; Rice, 1996). The viral genome of approximately 9600 nucleotides (nt) consists of a highly conserved 5' untranslated region (UTR), a single long open reading frame (ORF) of approximately 9000 nt, and a complex 3' UTR. The 5' UTR contains an internal ribosomal entry site (Tsukiyama-Kohara *et al.*, 1992; Honda *et al.*, 1996). The 3' UTR consists of a short variable region, a polypyrimidine tract of variable length, and, at the 3' end, a highly conserved region of approximately 100 nt (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995, 1996; Yamada *et al.*, 1996). The last 46 nucleotides of this conserved region were predicted to form a stable stem-loop structure thought to be critical for viral replication (Blight and Rice, 1997; Ito and Lai, 1997; Tsuchihara *et al.*, 1997). The ORF encodes a large polyprotein precursor that is cleaved into at least 10 proteins by host and viral proteinases (Rice, 1996). The predicted envelope proteins contain several conserved N-linked glycosylation sites and cysteine residues (Okamoto *et al.*, 1992a). The NS3 gene encodes a serine proteinase and an RNA helicase and the NS5B gene encodes an RNA-dependent RNA polymerase.

Globally, six major HCV genotypes (genotypes 1–6) and multiple subtypes (a, b, c, etc.) have been identified (Bukh *et al.*, 1993; Simmonds *et al.*, 1993). The most divergent HCV isolates differ from each other by more than 30% over the entire genome (Okamoto *et al.*, 1992a). Infection with genotype 1 is most prevalent (Bukh *et al.*, 1995). In the United States, HCV genotypes 1a and 1b constitute the majority of infections. In many other areas,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF054247–AF054268.

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especially in Europe and Japan, genotype 1b predominates. Recently, a number of studies have suggested that the severity of liver disease and the outcome of therapy may be genotype-dependent (reviewed in Bukh *et al.*, 1997). In these studies infection with HCV genotype 1b was associated with more severe liver disease (Brecht, 1997) and a poorer response to IFN therapy (Fried and Hoofnagle, 1995). However, the exact biological consequences of the genetic heterogeneity of HCV are still unclear.

HCV circulates in an infected individual as a quasi-species of closely related genomes (Bukh *et al.*, 1995; Farci *et al.*, 1997). The genetic heterogeneity of HCV is not distributed uniformly throughout the genome but is concentrated in hypervariable region 1 (HVR1) at the amino-terminal end of E2 (Weiner *et al.*, 1991; Hijikata *et al.*, 1991). The HVR1 region contains a neutralization epitope (Farci *et al.*, 1996; Shimizu *et al.*, 1996) and the amino acid sequence of HVR1 can undergo sequential changes during infection, probably resulting in escape from immune surveillance by the host and establishment of persistent HCV infection (Weiner *et al.*, 1992; Farci *et al.*, 1997). A second hypervariable region was identified in E2 (HVR2) but only in genotype 1b strains of HCV (Hijikata *et al.*, 1991). The quasispecies nature of HCV might also impact the severity of associated liver disease and resistance to IFN treatment (reviewed in Farci *et al.*, 1997).

Hepatitis C virus infects some continuous human T cell lines *in vitro* (Shimizu *et al.*, 1992) but replicates poorly in such cell cultures. The chimpanzee is the only nonhuman host of HCV (Farci *et al.*, 1993), but its availability is very limited. This lack of a convenient animal model or an efficient *in vitro* propagation system has made it difficult to study the virological characteristics of HCV or to develop antiviral therapies and vaccines.

Recently, two research groups independently constructed an infectious cDNA clone of strain H77 (genotype 1a) of HCV (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). In both studies, RNA transcripts encoding the consensus amino acid sequence of the putative polyprotein were infectious for chimpanzees, whereas those encoding a nonconsensus polyprotein were not. These infectious clones should aid in studying HCV replication and pathogenesis and should provide an important tool for development of *in vitro* replication and propagation systems. However, given the extensive genetic heterogeneity of HCV, it will probably be important to have infectious clones of more than one genotype. Although the near-complete genomic sequences of several HCV strains have been published, past experience suggests that it will be difficult to clone the infectious sequence. First, the viral quasispecies possibly include a proportion of defective or noninfectious genomes. Second, clinical samples obtained from patients or experimentally in-

fected chimpanzees contain only minute amounts of viral RNA. Therefore, it is necessary to perform *in vitro* amplification of viral RNA by reverse transcription (RT) and PCR, which can introduce errors into the cDNA derived from the viral genomes.

In the present study, we performed a detailed sequence analysis of the HC-J4 strain of genotype 1b that had been biologically amplified in a chimpanzee and constructed an infectious chimeric cDNA clone by inserting the complete ORF of the genotype 1b strain into a cassette vector that incorporated part of the untranslated regions of an infectious clone of a genotype 1a strain.

## RESULTS

### Quasispecies of strain HC-J4 in the infectious plasma pool used as the cloning source

An infectious cDNA clone of a genotype 1a strain of HCV had been obtained only after the ORF was engineered to encode the consensus polyprotein (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Thus, prior to constructing an infectious cDNA clone of a 1b genotype, we performed a detailed sequence analysis of the cloning source to determine the consensus sequence. A plasma pool of strain HC-J4 was prepared from acute-phase plasmapheresis units collected from a chimpanzee experimentally infected with HC-J4/91 (Okamoto *et al.*, 1992b). This HCV pool had a PCR titer of  $10^4$ – $10^5$  GE/ml and an infectivity titer of approximately  $10^3$  chimpanzee infectious doses/ml (Bukh *et al.*, unpublished data).

We determined the heterogeneity of the 3' UTR of strain HC-J4 by analyzing 24 clones of nested RT-PCR product. The consensus sequence was identical to that previously published for HC-J4/91 (Okamoto *et al.*, 1992b), except at position 9407 (see below). The variable region consisted of 41 nucleotides (nt 9372–9412), including two in-frame termination codons. Furthermore, its sequence was highly conserved except at positions 9399 (19 A and 5 T clones) and 9407 (17 T and 7 A clones). The poly(U-UC) region varied slightly in composition and greatly in length (31–162 nucleotides). In the conserved region, the first 16 nucleotides of 22 clones were identical to those previously published for other genotype 1 strains, whereas 2 clones each had a single point mutation. These data suggested that the structural organization at the 3' end of HC-J4 was similar to that of our infectious clone of a genotype 1a strain.

We next amplified the entire ORF of HC-J4 in a single round of long RT-PCR (Fig. 1). Our original plan was to clone the resulting PCR products into the *PinAI* and *BrlI* site of a HCV cassette vector (pCV), which had fixed 5' and 3' termini of genotype 1a (Yanagi *et al.*, 1997). However, we were not able to obtain full-length clones. There-

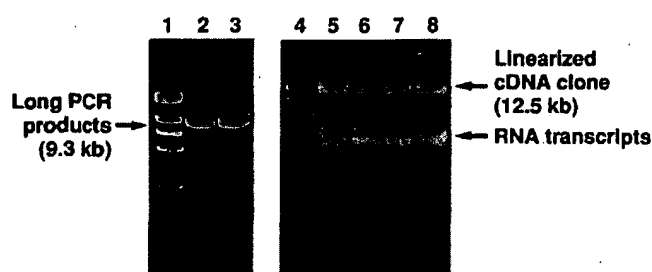


FIG. 1. Agarose gel of long RT-PCR amplicons and transcription mixtures. Lanes 1 and 4: Molecular weight marker ( $\lambda$ /HindIII digest). Lanes 2 and 3: RT-PCR amplicons of the entire ORF of HC-J4. Lane 5: pCV-H77C transcription control (Yanagi *et al.*, 1997). Lanes 6–8: 1/40 of each transcription mixture of pCV-J4L2S, pCV-J4L4S, and pCV-J4L6S, respectively, which was injected into the chimpanzee.

fore, we separately subcloned into pCV two genome fragments (L and S) derived from the long RT-PCR products (Fig. 2).

To determine the consensus sequence of the ORF we sequenced nine clones each of the L fragment (pCV-J4L) and of the S fragment (pCV-J4S). We found a quasispecies at 275 nucleotide (3.05%) and 78 amino acid (2.59%) positions, scattered throughout the 9030 nt (3010 aa) of the ORF (Fig. 3). Of the 161 nucleotide substitutions unique to a single clone, 71% were at the third position of the codon and 72% were silent.

Each of the nine L clones represented the near-complete ORF of an individual genome. The differences among the L clones were 0.30–1.53% at the nucleotide and 0.31–1.47% at the amino acid level (Fig. 4). Two clones, L1 and L7, had a defective ORF due to a single nucleotide deletion and a single nucleotide insertion, respectively. Even though the HC-J4 plasma pool was obtained in the early acute phase, it appeared to contain at least three viral species (Fig. 5). Species A contained the L1, L2, L6, L8, and L9 clones, species B the L3, L7, and L10 clones, and species C the L4 clone. Although each species A clone was unique, all A clones differed from all B clones at the same 20 amino acid sites (Fig. 3). At these positions, species C had the species A sequence at 14 positions and the species B sequence at 6 positions (Fig. 3).

Okamoto and co-workers (Okamoto *et al.*, 1992b) previously determined the nearly complete genome consensus sequence of strain HC-J4 in acute-phase serum of the first chimpanzee passage (HC-J4/83) as well as in chronic-phase serum collected 8.2 years later (HC-J4/91). In addition, they determined the sequence of amino acids 379 to 413 (including HVR1) and amino acids 468 to 486 (including HVR2) of multiple individual clones (Okamoto *et al.*, 1992b). We found that the sequences of individual genomes in the plasma pool collected from a chimpanzee inoculated with HC-J4/91 were all more closely related to HC-J4/91 than to HC-J4/83 (Figs. 4 and 5) and contained HVR amino acid sequences closely

related to three of the four viral species previously found in HC-J4/91 (Fig. 6).

#### The difficulty of determining the consensus sequence of HC-J4 in the plasma pool

We determined the consensus sequence of nucleotides 156–9371 of HC-J4 by two approaches. In one approach, the consensus sequence was deduced from nine clones of the long RT-PCR product. In the other approach the long RT-PCR product was reamplified by PCR as overlapping fragments which were sequenced directly. The two "consensus" sequences differed at 31 (0.34%) of 9216 nucleotide positions and at 11 (0.37%) of 3010 deduced amino acid positions (Fig. 3). At all of these positions a major quasispecies of strain HC-J4 was found in the plasma pool. At 9 additional amino acid positions the cloned sequences displayed heterogeneity and the direct sequence was ambiguous (Fig. 3). Finally, it should be noted that there were multiple amino acid positions at which the consensus sequence obtained by direct sequencing was identical to that obtained by cloning and sequencing even though a major quasispecies was detected (Fig. 3).

For positions at which the two "consensus" sequences of HC-J4 differed, we included both amino acids in a composite consensus sequence (Fig. 3). However, even with this allowance, none of the nine L clones analyzed (aa 1–2864) had the composite consensus sequence: two clones did not encode the complete polypeptide and the remaining seven clones differed from the consensus sequence by 3–13 amino acids (Fig. 3).

#### Chimeric full-length cDNA clones containing the entire ORF of HC-J4

Three full-length cDNA clones were constructed by cloning different L fragments into the *Pst*Al/*Bgl*II site of pCV-J4S9, the cassette vector for genotype 1a (Fig. 2), which also contained an S fragment encoding the consensus amino acid sequence of HC-J4. Therefore, although the ORF was from strain HC-J4, most of the 5' and 3' terminal sequence originated from strain H77 (Yanagi *et al.*, 1997). As a result, the 5' and 3' UTR were chimeras of genotypes 1a and 1b (Fig. 7). The first 155 nucleotides of the 5' UTR were from strain H77 (genotype 1a) and differed from the authentic sequence of HC-J4 (genotype 1b) at nucleotides 11, 12, 13, 34, and 35. In two clones (pCV-J4L2S, pCV-J4L6S) the rest of the 5' UTR had the consensus sequence of HC-J4, whereas the third clone (pCV-J4L4S) had a single nucleotide insertion at position 207. In all three clones the first 27 nucleotides of the 3' variable region of the 3' UTR were identical with the consensus sequence of HC-J4. The remaining 15 nucleotides of the variable region, the poly(U-UC) region, and the 3' conserved region of the 3' UTR had the same

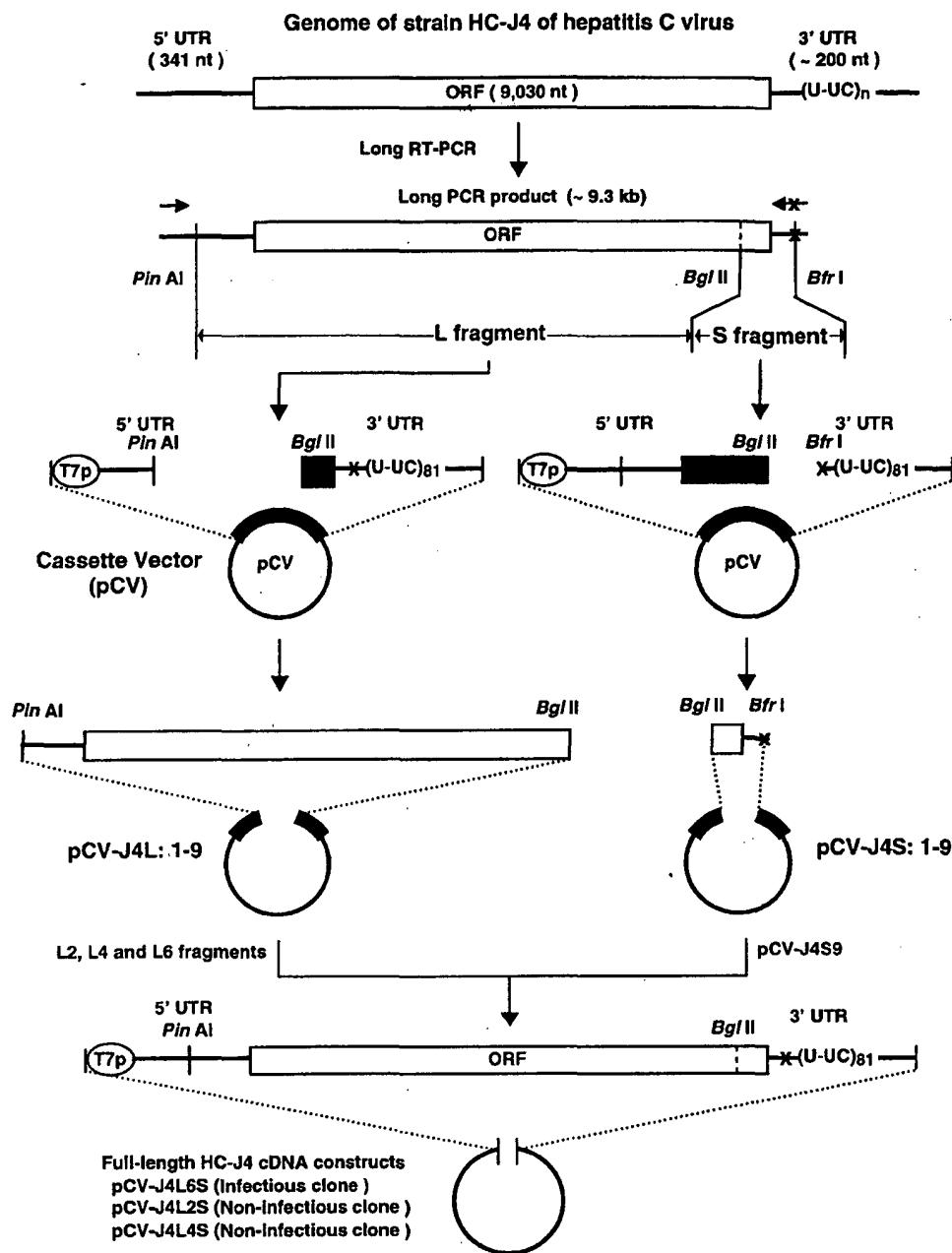


FIG. 2. Strategy for the construction of full-length cDNA clones of HCV strain HC-J4. The long PCR products were cloned as two separate fragments (L and S) into a cassette vector (pCV) with fixed 5' and 3' termini of HCV (Yanagi *et al.*, 1997). Full-length cDNA clones of HC-J4 were obtained by inserting the L fragment from three pCV-J4L clones into three identical pCV-J4S9 clones after digestion with *Pin*AI (isoschizomer of *Age*I) and *Bgl*II.

sequence as an infectious clone of strain H77 (Yanagi *et al.*, 1997).

None of the three full-length clones of HC-J4 had the ORF composite consensus sequence (Figs. 3 and 8). The pCV-J4L6S clone had only three amino acid changes: Q for R at position 231 (E1), V for A at position 937 (NS2), and T for S at position 1215 (NS3). The pCV-J4L4S clone had seven amino acid changes, including a change at position 450 (E2) that eliminated a highly conserved N-linked glycosylation site (Okamoto *et al.*, 1992a). Finally, the pCV-J4L2S clone had nine amino acid changes compared with the consensus sequence of HC-J4. A change at position 304 (E1) mutated a highly conserved

cysteine residue (Bukh *et al.*, 1993; Okamoto *et al.*, 1992a).

#### Transfection of a chimpanzee by transcripts from a chimeric cDNA clone

We tested the infectivity of RNA transcripts from the three chimeric cDNA clones simultaneously in a chimpanzee. The chimpanzee became infected with HCV as measured by increasing titers of  $10^2$  GE/ml at week 1 p.i.,  $10^3$  GE/ml at week 2 p.i., and  $10^4$ – $10^5$  GE/ml during weeks 3 to 11 p.i. (Fig. 9). The chimpanzee was negative for HCV RNA at weeks 19 and 20 p.i. (see Note added in



	L fragment	Cons-p9	L1* (A)	L2 (A)	L6 (A)	L8 (A)	L9 (A)	L3 (B)	L7* (B)	L10 (B)	L4 (C)	Cons-D	Cons-F
Core	16	N	S	.	.	.	.	.	.	.	.	.	N
	36	L	.	.	.	P	.	.	.	.	.	.	L
	52	A	.	.	.	.	.	F	Y	Y	T	T	A,T
	70	R	.	.	.	.	.	Q	Q	Q	.	R,Q	R,Q
E1	189	A	.	.	.	.	.	.	T	.	.	.	A
	195	R	.	.	.	.	.	H	.	H	.	.	R
	231	R	.	Q	Q	Q	.	.	.	.	.	.	R
	233	G	.	.	.	.	.	A	A	A	.	.	G
	234	N	.	.	.	.	.	D	D	D	.	.	N
	250	N	.	.	.	.	.	.	.	.	D	.	N
	299	E	A	.	.	.	A	.	.	.	.	.	E
	304	C	.	Y	.	.	.	.	.	.	.	.	C
E2	379	A	.	.	.	.	.	T	T	T	.	.	A
	384	E	.	.	.	.	.	T	T	T	.	E,T	E,T
	386	H	.	.	.	.	.	Y	Y	Y	.	H,Y	H,Y
	388	T	.	.	.	.	.	S	S	S	.	T,S	T,S
	390	R	.	.	.	.	.	G	G	G	.	G	R,G
	391	V	.	.	.	.	.	.	.	A	.	.	V
	392	A	V	.	.	V	V	.	.	.	V	V	A,V
	394	H	.	.	.	.	.	R	R	R	.	.	H
	405	S	.	.	.	.	.	.	P	.	.	.	S
	434	Q	.	.	.	.	.	H	H	H	.	H	Q,H
	438	F	.	.	.	.	.	L	L	L	.	L	F,L
	444	A	.	.	.	.	.	T	T	T	.	T	A,T
	450	S	.	.	.	.	.	.	.	.	P	.	S
	458	S	.	.	.	N	.	.	.	.	.	.	S
	466	A	.	.	.	.	.	V	V	V	.	A,V	A,V
	474	Y	.	.	.	.	.	H	.	.	.	.	Y
	476	K	.	.	.	.	.	E	E	E	.	E	K,E
	496	V	.	.	.	.	.	I	I	I	.	I	V,I
	524	V	.	.	.	.	A	.	A	.	.	.	V
	536	V	.	M	.	.	.	.	.	.	.	.	V
	580	I	.	.	.	.	.	.	V	.	.	.	I
	622	L	V	.	.	.	V	.	.	.	.	.	L
	673	Q	.	.	.	P	.	.	.	.	.	.	Q
p7	783	A	.	.	.	.	.	.	V	.	.	.	A
	820	G	.	.	.	.	.	.	S	.	.	.	G
	857	M	.	.	.	.	.	.	I	.	.	.	M
	927	K	.	.	.	.	.	.	R	.	.	.	K
NS2	934	V	I	I	.	I	I	.	.	.	.	.	V
	937	A	.	.	V	.	.	.	.	.	.	.	A
	978	A	.	.	.	.	.	D	D	D	.	D	A,D
	1028	P	.	.	.	.	S	.	.	.	.	.	P
NS3	1031	A	.	.	.	.	.	.	.	T	.	.	A
	1043	V	.	.	.	.	.	.	I	I	.	.	V,I
	1067	Q	.	.	.	.	.	H	H	H	.	Q,H	Q,H
	1097	I	X	.	.	.	.	.	.	.	.	.	I
	1188	G	R	.	.	.	.	.	.	.	.	.	G
	1215	S	.	.	T	.	.	.	.	.	.	.	S
	1223	F	.	S	.	.	.	.	.	.	.	.	F
	1226	A	.	.	.	.	.	.	.	V	.	.	A
	1339	A	V	.	.	.	.	.	.	.	.	.	A
	1399	K	N	.	.	.	.	.	.	.	.	.	K
	1503	T	.	.	.	.	.	S	.	S	.	.	T
	1528	Y	.	.	.	.	.	.	.	.	H	.	Y
	1535	T	A	.	.	.	.	.	.	.	.	.	T
NS4A	1662	L	.	P	.	.	.	.	.	.	.	.	L
	1753	K	.	R	.	.	.	.	.	.	.	.	K
	1805	H	.	.	N	.	.	N	.	N	N	N	H,N
NS4B	1949	S	.	.	.	.	.	.	.	.	P	.	S
	2105	M	.	.	.	.	V	I	.	I	.	.	M
	2138	K	.	.	.	.	.	.	.	.	R	.	K
	2146	T	.	.	.	.	.	A	A	A	.	T,A	T,A
	2226	L	.	.	.	.	.	P	.	.	.	.	L
	2259	L	.	.	.	.	.	F	.	.	.	.	L
	2262	E	.	.	.	.	.	D	D	D	.	E,D	E,D
	2334	V	.	.	.	.	.	I	.	.	.	.	V
	2371	L	.	.	.	.	.	Q	Q	Q	.	L,Q	L,Q
	2385	Y	.	.	.	.	.	.	.	.	H	.	Y
NS5B	2692	N	.	.	.	.	.	.	S	.	.	.	N
	2757	A	.	.	.	.	V	.	.	.	.	.	A
	2785	C	.	R	.	.	.	.	.	.	.	.	C
	2824	I	.	V	.	.	.	.	.	.	.	.	I
	2861	A	.	.	.	.	.	V	.	.	.	.	A
	S fragment		S5	S9	S2	S3	S7	S8	S10	S4	S6		
	2968	G	.	.	.	.	.	S	S	.	.	.	G
S fragment	2975	S	.	.	.	.	.	G	G	G	G	.	S
	2978	D	.	.	.	.	.	.	.	.	G	.	D
	2999	S	.	.	F	F	F	.	.	.	.	.	S

FIG. 3. Amino acid positions with a quasispecies of HC-J4 in the acute-phase plasma pool obtained from an experimentally infected chimpanzee. Cons-p9: consensus amino acid sequence deduced from analysis of nine L fragments and nine S fragments (see Fig. 2). Cons-D: consensus sequence derived from direct sequencing of the PCR product. A-C: groups of similar viral species. Dot: amino acid identical to that in Cons-p9. Capital letter: amino acid different from that in Cons-p9. Cons-F: composite consensus amino acid sequence combining Cons-p9 and Cons-D. Boxed amino acid: different from that in Cons-F. Shaded amino acid: different from that in all species A sequences. An asterisk indicates defective ORF due to a nucleotide deletion (clone L1, aa 1097) or insertion (clone L7, aa 2770). Diagonal lines: fragments used to construct the infectious clone.



nt \ aa	L1 (A)	L2 (A)	L6 (A)	L8 (A)	L9 (A)	L3 (B)	L7 (B)	L10 (B)	L4 (C)	HC-J4/91	HC-J4/83
L1 (A)		0.56	0.60	0.36	0.33	1.50	1.53	1.46	0.95	0.83	1.79
L2 (A)	0.59		0.55	0.45	0.50	1.49	1.51	1.45	0.98	0.82	1.77
L6 (A)	0.52	0.42		0.40	0.55	1.33	1.38	1.29	0.80	0.68	1.58
L8 (A)	0.42	0.38	0.31		0.31	1.32	1.34	1.28	0.79	0.65	1.62
L9 (A)	0.55	0.52	0.45	0.35		1.42	1.42	1.38	0.91	0.75	1.66
L3 (B)	1.47	1.43	1.15	1.33	1.36		0.61	0.30	1.43	0.90	1.51
L7 (B)	1.36	1.33	1.12	1.22	1.22	0.66		0.57	1.47	0.95	1.54
L10 (B)	1.36	1.33	1.05	1.22	1.26	0.31	0.56		1.37	0.85	1.42
L4 (C)	0.77	0.80	0.59	0.63	0.70	1.12	1.08	1.01		0.76	1.73
HC-J4/91	0.94	0.91	0.63	0.80	0.87	0.77	0.73	0.66	0.52		1.22
HC-J4/83	1.96	1.89	1.68	1.85	1.82	1.75	1.61	1.57	1.71	1.40	

FIG. 4. Comparisons (percent difference) of nucleotide (nt 156–8935) and predicted amino acid sequences (aa 1–2864) of L clones (species A–C, this study), HC-J4/91 (Okamoto *et al.*, 1992b), and HC-J4/83 (Okamoto *et al.*, 1992b). Differences among species A sequences and among species B sequences are shaded.

proof) and antibodies to HCV were detected (weeks 18–20 p.i.). Serum liver enzyme levels were within normal range throughout follow-up.

To identify which of the three full-length HC-J4 clones were infectious, we cloned and sequenced the NS3 region (nt 3659–4110) of HCV genomes amplified by RT-PCR from serum samples taken from the infected chimpanzee during weeks 2 and 4 p.i. The PCR primers were a complete match with each of the original three clones. Thus, this assay should not have preferentially amplified one virus over another. Sequence analysis of 26 and 24 clones obtained at weeks 2 and 4 p.i., respectively, demonstrated that all originated from the transcripts of pCV-J4L6S. The consensus sequence of PCR

products of the nearly complete genome (nt 11–9441), amplified from serum obtained during week 2 p.i., was identical to the sequence of pCV-J4L6S and there was no evidence of quasispecies. Thus, RNA transcripts of pCV-J4L6S, but not of pCV-J4L2S or pCV-J4L4S, were infectious *in vivo*.

The chimeric sequences of genotypes 1a and 1b in the UTRs were maintained in the infected chimpanzee. The consensus sequence of nucleotides 11–341 of the 5' UTR and the variable region of the 3' UTR, amplified from serum obtained during weeks 2 and 4 p.i., had the expected chimeric sequence of genotypes 1a and 1b (Fig. 7). Also, three of four clones of the 3' UTR obtained at week 2 p.i. had the chimeric sequence of the variable

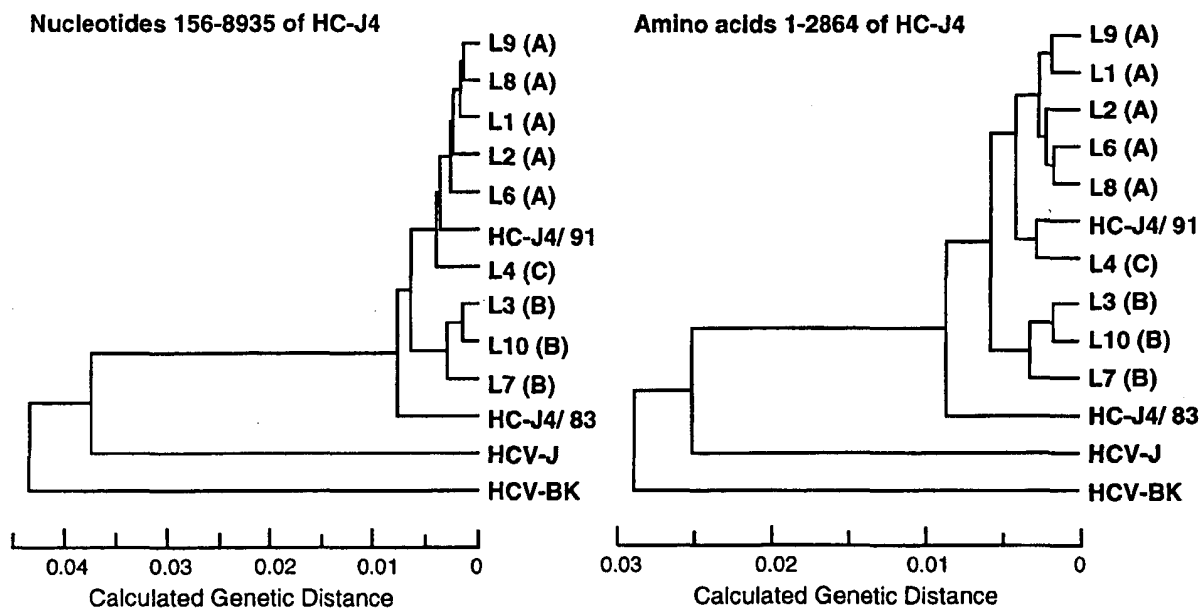


FIG. 5. UPGMA trees of HC-J4/91 (Okamoto *et al.*, 1992b), HC-J4/83 (Okamoto *et al.*, 1992b), two prototype strains of genotype 1b (HCV-J, Kato *et al.*, 1990; HCV-BK, Takamizawa *et al.*, 1991), and L clones (this study).

	379	413	468	486
HC-J4L6 (A) :	AGVDG	ETHTTGRVAGHTTSGFTSLFSSGAS QRIQL	GWGPIT	YTKPNSS DQRPYC
HC-J4L2 (A) :	.....	.....	.....	.....
HC-J4/91-20 :	.....	.....R.....	.....	.....E.....
HC-J4L1 (A) :	.....	.....V.....	.....	.....
HC-J4L8 (A) :	.....	.....V.....	.....	.....
HC-J4L9 (A) :	.....	.....V.....	.....	.....
HC-J4/91-21 :	.....	.....V.....	.....	.....G.....
HC-J4L4 (C) :	.....	.....V.R.....	.....	.....E.....
HC-J4/91-23 :	.....	.....V.R.....	.....	.....E.....
HC-J4/91-22 :	.....	.....V.R.....	.....	.....A.E.....
HC-J4L7 (B) :	.....	T.Y.S.G...R.....P.....	.....	.....E.....
HC-J4L10 (B) :	T....	T.Y.S.GA..R.....	.....	.....E.....
HC-J4L3 (B) :	T....	T.Y.S.G...R.....	.....	H.E.....
HC-J4/91-26 :	T....	T.Y.S.G...R.....	.....	G.D.L.....
HC-J4/91-25 :	.....	A.Y.S.G...R.....	.....	.....E.....
HC-J4/91-24 :	.....	A.Y.S.G...R.....	.....	.....E...P.....
HC-J4/91 :	.....	A.Y.S.G...R.....	.....	.....E...P.....
HC-J4/91-27 :	.....	K.Y.S.GA.S...R.....P...R...	.....	ESG.R.....
HC-J4/83 :	.....	Y.S.GA.S...TLA...P...R...	.....	E.D.P.....
	←-----→		←-----→	
	HVR1		HVR2	

FIG. 6. Alignment of the HVR1 and HVR2 amino acid sequences of the E2 of HCV. The sequences of nine L clones of HC-J4 (species A-C) obtained from an early acute-phase plasma pool of an experimentally infected chimpanzee were compared with the sequences of eight clones (HC-J4/91-20 through HC-J4/91-27, Okamoto *et al.*, 1992b) derived from the inoculum. Dot: an amino acid identical to that in the top line. Capital letters: amino acid different from that in the top line.

region, whereas a single substitution was noted in the fourth clone. However, in all four clones the poly(U) region was longer (2–12 nt) than expected. Also, we observed extra C and G residues in this region. For the most part, the number of C residues in the poly(UC) region was maintained in all clones, although the spacing varied. As we showed previously, variations in the number of U residues can reflect artifacts introduced during PCR amplification (Yanagi *et al.*, 1997). The sequence of the first 19 nucleotides of the conserved region was maintained in all four clones. Thus, with the exception of the poly(U-UC) region, the genomic sequences recovered from the infected chimpanzee were exactly those of the chimeric infectious clone.

## DISCUSSION

After much effort, the first cDNA clones of HCV that are infectious for chimpanzees were recently constructed (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Both of these previously reported clones were derived from strain H77, which is genotype 1a. We used the cassette vector we developed to clone strain H77 to construct an infectious cDNA clone containing the ORF of a second subtype. This new clone contains a chimeric HCV genome, which is composed mostly of genotype 1b sequences from strain HC-J4. The encoded polyproteins of genotypes 1a and 1b share only about 85% identity. Genotype 1b is the most prevalent genotype of HCV in the United States, Europe, and Japan. The availability of infectious clones

representing two important subtypes of genotype 1 should provide new ways of studying this virus.

There is no effective *in vitro* propagation or replication system for HCV. Thus, the infectivity of HCV clones has been determined by *in vivo* transfection: viral nucleic acid is injected directly into the liver of a chimpanzee. The previous transfection protocols had required laparotomy (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997), which can be performed only once per animal. In the present study, we demonstrated that the *in vivo* transfection could be performed by ultrasound-guided percutaneous intrahepatic injection (St. Claire *et al.*, unpublished data). This less invasive procedure should facilitate *in vivo* studies of cDNA clones of HCV in chimpanzees, since percutaneous procedures can be performed repeatedly.

We demonstrated that RNA transcripts of one cDNA clone of HC-J4, but not of two other clones with different ORFs but the same termini, were infectious *in vivo*. We injected the same amount of cDNA and transcription mixture for each of the clones (Fig. 1) so the failures to infect were not due to insufficient RNA. In the previous two reports on infection of chimpanzees only those clones engineered to have the independently determined and slightly different consensus amino acid sequence of the polyprotein of strain H77 were infectious (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Although the two infectious clones differed at 4 amino acid positions, these differences were represented in a major component of the quasispecies of the cloning source (Yanagi *et*

[illegible]

3' untranslated Region

3' variable region

poly U-UC region

3' conserved region

9372

9513

HC-J4 : TGAACGGGGA GCTAACCACT CCAGGCCAAT AGGCCCTT--C CTG Poly (U-UC)<sub>n</sub> ---GGTGGCT CCATCTTAG

pCV-J4L6S: .....-T..A..A.TT. ... Poly (U-UC)<sub>81</sub> AAT.....

pCV-H77C : ...G.TT.G .G...A... .G.C.TCT..A..A.TT. ... Poly (U-UC)<sub>81</sub> AAT.....

Bfr1

3' conserved region (cont.)

9514

9595

H77 : CCCTAGTCAC GGCTAGCTGT GAAAGGTCGG TGAGCCGCAT GACTGCAGAG AGTGCTGATA CTGGCCTCTC TGCAGATCAT GT

pCV-J4L6S: ..... . . . . .

pCV-H77C : ..... . . . . .

FIG. 7. Alignment of the 5' and the 3' UTR sequences of infectious clones of genotype 1a (pCV-H77C) and 1b (pCV-J4L6S). Top line: consensus sequence of the indicated strain. Dot: identity with consensus sequence. Capital letter: different from the consensus sequence. Dash: deletion. Underlined: *PinA*I and *Bf*I cleavage site. Numbering corresponds to the HCV sequence of pCV-J4L6S.

*al.*, unpublished data) and therefore did not represent substantial differences. In the present study, we were unable to define a single consensus sequence of strain HC-J4, because the consensus sequence obtained by two different approaches (direct sequencing and sequencing of cloned products) differed at 20 amino acid positions, even though the same genomic PCR product was analyzed. The infectious clone differed at 2 positions

from the composite amino acid consensus sequence, from the sequence of the eight additional HC-J4 clones analyzed in this study and from published sequences of earlier passage samples. An additional amino acid differed from the composite consensus sequence but was found in two other HC-J4 clones analyzed in this study. The two noninfectious full-length clones of HC-J4 differed from the composite consensus sequence by only 7 and

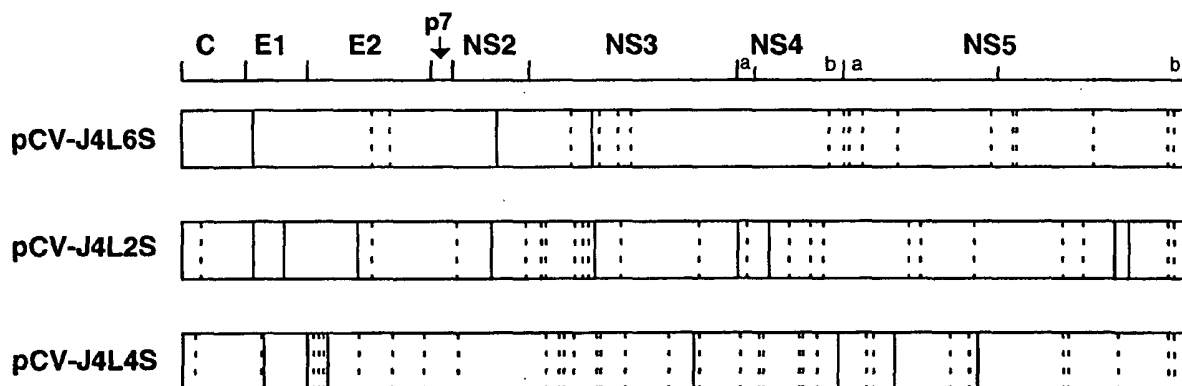


FIG. 8. Comparison of the ORF of individual full-length cDNA clones of HCV strain HC-J4 with the consensus sequence (see Fig. 3). Solid lines: amino acid changes. Dashed lines: silent mutations. Clone pCV-J4L6S was infectious *in vivo*, whereas clones pCV-J4L2S and pCV-J4L4S were not.

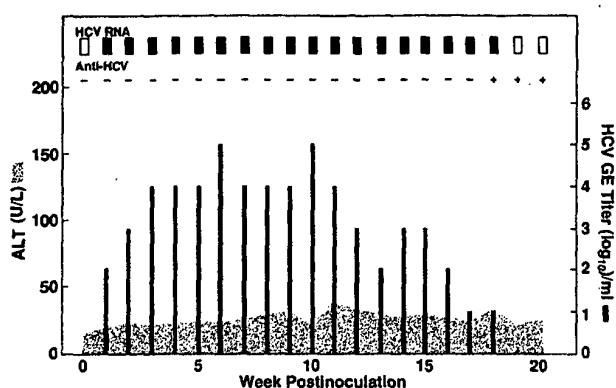


FIG. 9. Course of infection with hepatitis C virus (HCV) in chimpanzee 1500 following transfection with the infectious cDNA clone pCV-J4L6S (strain HC-J4, genotype 1b). Results of qualitative RT-nested PCR for HCV RNA (filled rectangles, positive; empty rectangles, negative) and second-generation ELISA test for anti-HCV [(+) positive; (–) negative] are shown. Serum levels of alanine aminotransferase (ALT; shaded area) and the log<sub>10</sub> HCV genome equivalent titer (vertical columns) are plotted against time.

9 amino acid differences. However, since these clones had the same termini as the infectious clone (except for a single nucleotide insertion in the 5' UTR of pCV-J4L4S), one or more of these amino acid changes in each clone was apparently deleterious for the virus. Although we have formally demonstrated for the first time that HCV polyprotein sequences other than the consensus sequence can be infectious, our data confirmed that deviations from the consensus sequence are often lethal.

The genome termini must interact with the viral polymerase during initiation of viral replication. Since the amino acid sequence of the putative RNA-dependent RNA polymerases of HC-J4 and H77 varied by more than 10% it was not obvious that the polymerase of HC-J4 would be able to replicate a chimeric genome containing portions of the H77 termini. The 5' UTR sequence of the infectious genotype 1b clone differed from the sequence of the original genotype 1b virus at 5 nucleotide positions, all close to the 5' end where they might be expected to affect RNA replication. In the 3' UTR of this infectious clone, the 3' variable sequence was chimeric and represented strain H77 rather than strain HC-J4 in 5 of 42 positions. Thus, although the 3' variable sequence was highly conserved within HC-J4, this strain-specific sequence was not critical for replication by the HC-J4 polymerase. In the present study, we found that HC-J4, like other strains of genotype 1b (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996; Yamada *et al.*, 1996), had a poly(U-UC) region followed by a terminal conserved element. The poly(U-UC) region appears to vary considerably, so it was not clear whether changes in this region would have a significant effect on virus replication. On the other hand, the 3' 98 nucleotides of the HCV genome were previously shown to be identical among other strains of genotypes 1a and 1b (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996). Thus, use of the cassette vector would not alter

this region except for addition of 3 nucleotides found in strain H77 between the poly(UC) region and the 3' 98 conserved nucleotides. This study showed for the first time that it is possible to make infectious viruses containing terminal sequences specific for two different subtypes of the same major genotype of HCV.

The viremic pattern found in the early phase of the infection with the recombinant HC-J4 virus was similar to that observed for the recombinant H77 virus in chimpanzees (Bukh *et al.*, unpublished data; Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Two chimpanzees infected with the recombinant H77 virus developed hepatitis (Bukh *et al.*, unpublished data), whereas the chimpanzee infected with the recombinant HC-J4 virus in the present study had no biochemical evidence of hepatitis. It is well known that biological variation exists and some chimpanzees inoculated with HCV either lack or develop minimal evidence of hepatitis even though the same inoculum caused disease in other animals. Thus, the phenotype of the infectious clone of strain HC-J4 can be determined only by transfection of several chimpanzees.

The chimpanzee transfected in the present study was chronically infected with hepatitis G virus (HGV/GBV-C) (Bukh *et al.*, 1998) and had a titer of 10<sup>6</sup> GE/ml at the time of HCV transfection. Although HGV/GBV-C was originally believed to be a hepatitis virus, it does not cause hepatitis in chimpanzees (Bukh *et al.*, 1998) and may not replicate in the liver (Laskus *et al.*, 1997). Our study demonstrated that an ongoing infection of HGV/GBV-C did not prevent acute HCV infection in the chimpanzee model.

In two previous studies it was reported that RNA transcripts from cDNA clones of HCV-1 (genotype 1a) and HCV-N (genotype 1b), respectively, resulted in viral replication after transfection into human hepatoma cell lines (Dash *et al.*, 1997; Yoo *et al.*, 1995). In both of these studies, infectivity was reported for clones that did not contain the terminal 98 conserved nucleotides at the very 3' end of the 3' UTR. The viability of these clones was not tested *in vivo* and concerns were raised about the infectivity of these cDNA clones *in vitro* (Fausto, 1997). With the chimpanzee transfection system we have developed, we have begun to test directly the requirement for this region in a biologically relevant way.

Since the nearly complete ORF was amplified in long RT-PCR and cloned in one step, we were able to study the quasispecies nature of the cloning source as reflected by colinear mutations throughout the HCV genome. Our analysis showed that the quasispecies of HC-J4 found in this acute-phase pool represented at least three viral species and that a quasispecies in the HVR1 region of HCV paralleled quasispecies at multiple other positions throughout the genome. It was previously demonstrated that each chimpanzee inoculated with an acute-phase plasma pool of patient H (H77) became infected with a different dominant sequence (Farci *et al.*,

1994), indicating that multiple components of a quasi-species were infectious. Our findings in the present study indicate that simultaneous transmission of multiple species to a single chimpanzee occurred. The quasiespecies of HC-J4 found in the acute-phase pool represented three of the four major quasiespecies found in the inoculum (HC-J4/91, Okamoto *et al.*, 1992b). Our observation clearly illustrates the difficulties in accurately determining the evolution of HCV over time since multiple species with significant changes throughout the HCV genome can be present from the onset of the infection. Infection of chimpanzees with monoclonal viruses derived from the infectious clones should make it possible to perform more detailed studies of the evolution of HCV *in vivo* and its importance for viral persistence and pathogenesis.

In conclusion, we have constructed an infectious clone representing a genotype 1b strain of HCV. Our study demonstrated that it was possible to obtain an infectious clone of a second strain of HCV, that a consensus amino acid sequence was not absolutely required for infectivity, and that chimeras between the UTRs of two different genotypes could be viable. The availability of an infectious clone of a second strain of HCV, representing the most prevalent genotype in the world, should be important for further studies of viral replication and pathogenesis of HCV and should permit more detailed studies of the function of HCV proteins.

## MATERIALS AND METHODS

### Source of HCV genotype 1b

An infectious plasma pool (second chimpanzee passage) containing strain HC-J4, genotype 1b, was prepared from acute phase plasma of a chimpanzee experimentally infected with serum containing HC-J4/91 (Okamoto *et al.*, 1992b). The HC-J4/91 sample was obtained from a first chimpanzee passage during the chronic phase of hepatitis C about 8 years after experimental infection. The consensus sequence of the entire genome, except for the very 3' end, was determined previously for HC-J4/91 (Okamoto *et al.*, 1992b).

### Preparation of HCV RNA

Viral RNA was extracted from 100- $\mu$ l aliquots of the HC-J4 plasma pool with the TRIzol system (GIBCO BRL). The RNA pellets were each resuspended in 10  $\mu$ l of 10 mM dithiothreitol (DTT) with 5% (vol/vol) RNasin (20–40 units/ $\mu$ l) (Promega) and stored at  $-80^{\circ}\text{C}$  or immediately used for cDNA synthesis.

### Amplification and cloning of the 3' UTR

A region spanning from NS5B to the conserved region of the 3' UTR was amplified in nested RT-PCR (Yanagi *et al.*, 1997). The RNA was denatured at  $65^{\circ}\text{C}$  for 2 min, and cDNA was synthesized at  $42^{\circ}\text{C}$  for 1 h with Superscript

II reverse transcriptase (GIBCO BRL) and primer H3'X58R (Yanagi *et al.*, 1997) in a 20- $\mu$ l reaction volume. The cDNA mixture was treated with RNase H and RNase T1 (GIBCO BRL) at  $37^{\circ}\text{C}$  for 20 min. The first round of PCR was performed on 2  $\mu$ l of the final cDNA mixture in a total volume of 50  $\mu$ l with the Advantage cDNA polymerase mix (Clontech) and external primers H9261F and H3'X58R (Yanagi *et al.*, 1997). In the second round of PCR [internal primers H9282F and H3'X45R (Yanagi *et al.*, 1997)], 5  $\mu$ l of the first round PCR mixture was added to 45  $\mu$ l of the PCR reaction mixture. Each round of PCR (35 cycles) was performed in a DNA thermal cycler 480 (Perkin-Elmer) and consisted of denaturation at  $94^{\circ}\text{C}$  for 1 min (first cycle: 1 min 30 s), annealing at  $60^{\circ}\text{C}$  for 1 min, and elongation at  $68^{\circ}\text{C}$  for 2 min. After purification with QIAquick PCR purification kit (QIAGEN), digestion with *Hind*III and *Xba*I (Promega), and phenol/chloroform extraction, the amplified products were cloned into pGEM-9zf(–) (Promega) (Yanagi *et al.*, 1997).

### Amplification and cloning of the entire ORF

A region from within the 5' UTR to the variable region of the 3' UTR of strain HC-J4 was amplified by long RT-PCR (Fig. 1) (Yanagi *et al.*, 1997). The cDNA was synthesized at  $42^{\circ}\text{C}$  for 1 h in a 20- $\mu$ l reaction volume with Superscript II reverse transcriptase and primer J4-9405R (5'-GCCTATTGGCCTGGAGTGGTTAGCTC-3') and treated with RNases as above. The cDNA mixture (2  $\mu$ l) was amplified by long PCR with the Advantage cDNA polymerase mix and primers A1 (Bukh *et al.*, 1992; Yanagi *et al.*, 1997) and J4-9398R (5'-AGGATGGCCTTAAGGCCTGGAGTGGTTAGCTCCCCGTTCA-3'). Primer J4-9398R contained extra bases (*italics*) and an artificial *Afl*II cleavage site (underlined). A single PCR round was performed in a Robocycler thermal cycler (Stratagene) and consisted of denaturation at  $99^{\circ}\text{C}$  for 35 s, annealing at  $67^{\circ}\text{C}$  for 30 s, and elongation at  $68^{\circ}\text{C}$  for 10 min during the first 5 cycles, 11 min during the next 10 cycles, 12 min during the following 10 cycles, and 13 min during the last 10 cycles.

After we digested the long PCR products obtained from strain HC-J4 with *Pin*AI (isoschizomer of *Age*I) and *Bfr*I (isoschizomer of *Afl*II) (Boehringer Mannheim), we attempted to clone them directly into a cassette vector (pCV), which contained the 5' and 3' termini of strain H77 (Yanagi *et al.*, 1997). However, we did not obtain any full-length clones. To improve the efficiency of cloning, we further digested the PCR product with *Bgl*II (Boehringer Mannheim) and cloned the two resultant genome fragments (L fragment: *Pin*AI/*Bgl*II, nt 156–8935; S fragment: *Bgl*II/*Bfr*I, nt 8936–9398) separately into pCV (Fig. 2). DH5 $\alpha$ -competent cells (GIBCO BRL) were transformed and selected on LB agar plates containing 100  $\mu$ g/ml ampicillin (SIGMA) and amplified in LB liquid cultures at  $30^{\circ}\text{C}$  for 18–20 h. Sequence analysis of nine

plasmids containing the S fragment (miniprep samples) and nine plasmids containing the L fragment (maxiprep samples) were performed as described previously (Yanagi *et al.*, 1997).

Three L fragments, each encoding a distinct polyprotein, were cloned into pCV-J4S9, which contained an S fragment encoding the consensus amino acid sequence of HC-J4, to construct three chimeric full-length HCV cDNAs (pCV-J4L2S, pCV-J4L4S, and pCV-J4L6S) (Fig. 2). Large-scale preparation of each clone was performed as described previously (Yanagi *et al.*, 1997) and the authenticity of each clone was confirmed by sequence analysis.

### Sequence analysis

Both strands of DNA were sequenced with the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit using *Taq* DNA polymerase (Perkin-Elmer) and about 90 specific sense and antisense primers. Analyses of genomic sequences, including multiple sequence alignments and tree analyses, were performed with GeneWorks (Oxford Molecular Group) (Bukh *et al.*, 1995).

We determined the consensus sequence of strain HC-J4 by direct sequencing of PCR products (nt 11–9412) and by sequence analysis of multiple cloned L and S fragments (nt 156–9371). The consensus sequence of the 3' UTR (the 3' variable region, the polypyrimidine tract, and the first 16 nt of the conserved region) was determined by analysis of 24 cDNA clones.

### Intrahepatic transfection of a chimpanzee with transcribed RNA

Two *in vitro* transcription reactions were performed with each of the three full-length clones. In each reaction 10 µg of plasmid DNA linearized with *Xba*I (Promega) was transcribed in a 100-µl reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 h as described previously (Yanagi *et al.*, 1997). Five microliters of the final reaction mixture was analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 1). Each transcription mixture was diluted with 400 µl of ice-cold phosphate-buffered saline without calcium or magnesium and then the two aliquots from the same cDNA clone were combined, immediately frozen on dry ice, and stored at –80°C. Within 24 h after freezing the transcription mixtures were injected into the chimpanzee by percutaneous intrahepatic injection that was guided by ultrasound. Each inoculum was individually injected (five or six sites) into a separate area of the liver to prevent complementation or recombination. The chimpanzee was maintained under conditions that met all requirements for its use in an approved facility.

Serum samples were collected weekly from the chimpanzee and monitored for liver enzyme levels [alanine aminotransferase (ALT), gammaglutamyltranspeptidase (GGT), and isocitrate dehydrogenase (ICD)] and anti-HCV

antibodies [second-generation ELISA test (Abbott)]. Weekly samples of 100 µl of serum were tested for HCV RNA in a sensitive nested RT-PCR assay (Bukh *et al.*, 1992, Yanagi *et al.*, 1996) with AmpliTaq Gold DNA polymerase. The genome equivalent (GE) titer of HCV was determined by testing 10-fold serial dilutions of the extracted RNA in the RT-PCR assay (Yanagi *et al.*, 1996). We defined one GE as the number of HCV genomes present in the highest dilution positive in the RT-nested PCR assay. Duplicate titers determined by the "Amplicor HCV Monitor Test" (Roche Diagnostic Systems) (data not shown) were equivalent to these except at weeks 2, 17, and 18 p.i., where the titers were below the detection limit of the Amplicor HCV Monitor Test.

To identify which of the three clones was infectious *in vivo* we amplified the NS3 region (nt 3659–4110) from the chimpanzee serum in a highly sensitive and specific nested RT-PCR assay with AmpliTaq Gold DNA polymerase and cloned the PCR products with a TA cloning kit (Invitrogen). In addition, the consensus sequence of the nearly complete genome (nt 11–9441) was determined by direct sequencing of overlapping PCR products.

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*Note added in proof.* Following submission of this manuscript we found that chimpanzee 1500 was again positive for HCV RNA during weeks 21–24 p.i. (viral titer < 10<sup>3</sup> GE/ml). Thus we cannot determine at this time whether the infection with the chimeric recombinant 1b virus is chronic.

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